



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US91/03660 <b>(22) International Filing Date:</b> 3 June 1991 (03.06.91) <b>(30) Priority data:</b> 533,225 4 June 1990 (04.06.90) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 533,225 (CON) Filed on 4 June 1990 (04.06.90) <b>(71) Applicant (for all designated States except US):</b> SCHERING CORPORATION [US/US]; 2000 Galloping Hill Road, Kenilworth, NJ 07033 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> REICHERT, Paul [US/US]; 11 Cambray Road, Montville, NJ 07045 (US). HAMMOND, Gerald, S. [US/US]; 80 Arsdale Terrace, East Orange, NJ 07018 (US). LE, Hung, V. [US/US]; 75 Valley View Drive, Rockaway, NJ 07866 (US). NAGAB-HUSHAN, Tattanahalli, L. [US/US]; 3 Sunset Lane, Parsippany, NJ 07054 (US). TROTTA, Paul, P. [US/US]; 2429 Harmon Cove Towers, Secaucus, NJ 07094 (US).	<b>(74) Agents:</b> NELSON, James, R. et al.; Schering-Plough Corporation, One Giralda Farms, Madison, NJ 07940-1000 (US). <b>(81) Designated States:</b> AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), DE, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> METHOD FOR PREPARING INTERFERON ALPHA-2 CRYSTALS		
<b>(57) Abstract</b>  A method for making crystals interferon alpha-2 the use thereof in depot formulations are disclosed.		

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5        **METHOD FOR PREPARING INTERFERON ALPHA-2**  
         **CRYSTALS**

10

**BACKGROUND OF THE INVENTION**

         The human interferon alphas are a family of proteins  
15   comprising at least 24 subspecies, Zoon K.C. , Interferon 9, 1-12 (1987) ,  
         Gresser I., ed. Academic Press, New York. They were originally  
         described as agents capable of inducing an antiviral state in cells but  
         are known as pleiotropic lymphokines affecting many functions of the  
         immune system, Opdenakker, et al., Experimentia 45, 513-520 (1989).  
20   Apart from their *in vitro* biological activities the human interferon alphas  
         are currently used for several important indications, e.g. hairy cell  
         leukemia, Kaposi Sarcoma, venereal warts, and are being investigated  
         for several others Intron A (interferon alpha-2b) Clinical status (1989).  
         Proceedings from a satellite symposium at the 5<sup>th</sup> European  
25   Conference on Clinical Oncology, London, U.K. September 1989. The  
         demand for highly purified and crystalline forms of interferon alpha,  
         especially the recombinant type alpha-2b is of foremost importance for  
         structure elucidation as well as for formulation of various dosage forms.

         Two forms of crystalline human interferon alpha-2 have  
30   been reported. Miller et al., Science, 215, 689-690 (1982); Kung et al.,  
         U.S. Patent No. 4,672,108; Weissmann, The Cloning of Interferon and  
         other Mistakes, In: Interferon 1981, Ion Gresser, ed., Academic Press,  
         New York, 101-134; Weissmann, Phil. Trans. R. Soc. Lond., B299, 7-28  
         (1982); and Nagabhusban, et al., Characterization of Genetically  
35   Engineered Alpha-2 Interferon, In: Interferon: Research, Clinical

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Application and Regulatory Consideration, Zoon, et al., Elsevier, New York 79-88 (1982). These publications describe methods for crystallizing interferon alpha-2 from polyethylene glycol at low temperature or from a phosphate buffer solution by adjusting the pH or temperature. These methods normally provide needle crystals which cannot be well characterized by X-ray diffraction techniques. The Miller et al. article also mentions crystalline Interferon alpha-2 in a "prismatic form".

In general, the methods for crystallizing proteins such as interferon have been found to be unpredictable. For example, Kung et al., U.S. Patent No. 4,672,108 (1987) specifically states in column 1, lines 52-64:

"Numerous techniques have been developed for the crystallization of proteins, however, no generalized procedure has been discovered, and many proteins remain uncrystallized. Thus, crystallization of proteins is an unpredictable art utilizing trial and error procedures among many possible alternative methodologies.

One of the most widely used approaches involves the addition to the protein solution of a crystallizing agent, which is commonly a salt, such as ammonium sulfate or ammonium citrate or an organic solvent, such as ethanol or 2-ethyl-2,4-pentenediol. However, such procedures do not provide a suitable means for producing crystalline human leukocyte interferons." (Emphasis added.)

#### SUMMARY OF THE INVENTION

We have now surprisingly found that high quality crystalline interferon alpha-2 can be produced efficiently even at room temperature by a method which comprises equilibrating a solution of interferon alpha-2 against a sulfate salt solution that will cause the interferon alpha-2 solution to become more concentrated and form interferon alpha-2 crystals. Preferably, the equilibration is effected by

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means of ultrafiltration or dialysis, or using drops, e.g., by hanging or sandwiched droplets.

The solution of interferon alpha-2 preferably contains a sulfate salt, and this solution is preferably equilibrated against a more concentrated sulfate salt solution. The sulfate salt is preferably selected from an ammonium, calcium, cadmium, potassium, lithium, magnesium or sodium salt, more preferably it is ammonium sulfate. The sulfate salt is preferably present in the crystalline interferon alpha-2 solution at the time crystals begin to form in a concentration of from about 12% to about 30% saturated, more preferably in a concentration of from about 15% to about 25% saturated ammonium sulfate. As noted below, the concentration of sulfate salt at the start of the equilibration procedure will be lower, i.e., from about 2% to about 18% saturated.

Preferably, the interferon-alpha-2 is interferon alpha-2b and is more preferably human, recombinant crystalline interferon alpha-2b. In one embodiment, the crystalline material is interferon alpha-2b having the amino acid sequence shown below:

	1	Cys	Asp	Leu	Pro	Gln	Thr	His	Ser	Leu	Gly	Ser	Arg
20	13	Arg	Thr	Leu	Met	Leu	Leu	Ala	Gln	Met	Arg	Arg	Ile
	25	Ser	Leu	Phe	Ser	Cys	Leu	Lys	Asp	Arg	His	Asp	Phe
	37	Gly	Phe	Pro	Gln	Glu	Glu	Phe	Gly	Asn	Gln	Phe	Gln
	49	Lys	Ala	Glu	Thr	Ile	Pro	Val	Leu	His	Glu	Met	Ile
	61	Gln	Gln	Ile	Phe	Asn	Leu	Phe	Ser	Thr	Lys	Asp	Ser
25	73	Ser	Ala	Ala	Trp	Asp	Glu	Thr	Leu	Leu	Asp	Lys	Phe
	85	Tyr	Thr	Glu	Leu	Tyr	Gln	Gln	Leu	Asn	Asp	Leu	Glu
	97	Ala	Cys	Val	Ile	Gln	Gly	Val	Gly	Val	Thr	Glu	Thr
	109	Pro	Leu	Met	Lys	Glu	Asp	Ser	Ile	Leu	Ala	Val	Arg
	121	Lys	Tyr	Phe	Gln	Arg	Ile	Thr	Leu	Tyr	Leu	Lys	Glu
30	133	Lys	Lys	Tyr	Ser	Pro	Cys	Ala	Trp	Glu	Val	Val	Arg
	145	Ala	Glu	Ile	Met	Arg	Ser	Phe	Ser	Leu	Ser	Thr	Asn
	157	Leu	Gln	Glu	Ser	Leu	Arg	Ser	Lys	Glu			

Interferon alpha-2a may also be employed. The primary amino acid sequence of interferon alpha-2a differs from the above

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sequence of interferon alpha-2b by the replacement of lysine for arginine at residue position 23.

The sulfate salt solution of interferon alpha-2 preferably includes a buffer having a pH of 6.5 to 8.5, more preferably from 7.3 to 8.0, such as a sodium phosphate buffer solution.

The crystalline interferon alpha-2 prepared by the above methods may be used as seed crystals for preparing additional crystalline interferon alpha-2.

#### 10 DETAILED DESCRIPTION OF THE INVENTION

As noted above, the method of the invention involves equilibrating a sulfate salt solution of interferon alpha-2 against a sulfate salt solution that will cause the crystalline interferon alpha-2 solution to become more concentrated and form interferon alpha-2 crystals. Any suitable interferon alpha-2 can be employed, e.g., interferon alpha-2a and interferon alpha-2b, more preferably human, recombinant interferon alpha-2a (r-h-interferon alpha-2a) or interferon alpha-2b (r-h-interferon alpha-2b). Commercially available alpha-2 interferon preparations are available from Hoffmann-La Roche (Roferon®) and Schering-Plough (Intron®A). Mixtures of pure interferons including alpha-2 interferons are available from Burroughs-Wellcome Corporation (Wellferon®). In view of the high degree of sequence homology in the human interferon-alphas, the method of the present invention should be applicable for each subspecies.

The human interferon alpha-2 subspecies may be obtained through recombinant DNA technology or may be purified from natural sources (e.g. human peripheral blood lymphocytes, human lymphoblastoid cell lines), for example, as described in Pestka, et al., Ann. Rev. Biochem. 56, 727-777 (1987). A preferred interferon alpha-2 is r-h-interferon alpha-2b having the amino acid sequence as set forth above.

Natural human interferon alphas have been purified from several cell sources including leukocytes isolated from whole blood, neonatal fibroblasts, lymphoblastoid and various leukemic cell lines.

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The first clinically available preparation of human leukocyte interferon was developed by K. Cantell and associates in Finland, centrifuged blood from normal donors is primed with interferon, induced by addition of Sendai virus and centrifuged. The resulting supernatant is

5 precipitated with potassium thiocyanate, extracted with ethanol, pH precipitated, and dialyzed against phosphate-buffered saline (K.E. Morgensen, L. Cantell (1977) Pharmacol. Ther. 1, 369-381).

Recombinant interferon alphas have been cloned and expressed in E. coli by several groups, for example, C. Weissmann, et

10 al. Science 209 (1980) 1343-1349. The purification of recombinant alpha-2 interferons has been described by several groups using a combination of chromatographic steps such as ammonium sulfate precipitation, dye affinity chromatography, ion exchange and gel filtration, for example, as described in Weissmann, C., Phil R. Soc.

15 (Lond), (1982) b299, 7-28). An alternative approach for purifying recombinant interferon alphas employs immunoaffinity chromatography with an immobilized antibody (P.P. Trotta et al., Developments in Industrial Microbiology 72, Elsevier, Amsterdam (1987) 53-64). For a review of available purification schemes used for recombinant alpha

20 interferons, see T.L. Nagabhushan and P.P. Trotta, Ullmann's Encyclopedia of Industrial Chemistry. A14, VCH, Weinheim, Federal Republic of Germany (1989) 372-374. Preferably, the interferon alpha-2b used is purified by a conventional purification process described in Ullmann's Encyclopedia of Industrial Chemistry. A14, VCH, Weinheim,

25 Federal Republic of Germany (1989) 372-374 followed by reversed phase high performance chromatography.

Suitable methods of equilibration include dialysis, ultrafiltration, e.g. diafiltration, or using drops, e.g., hanging or sandwiched droplets. Equilibration can be effected against a second

30 sulfate salt solution that is more concentrated than the sulfate salt solution of interferon alpha-2. A particularly preferred method is to equilibrate a solution of r-h-interferon alpha-2 against a sulfate salt solution using a phosphate buffer solution. Preferably, the equilibration occurs slowly, e.g., over 2 to 30 days.

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Large scale crystallization may be accomplished by methods equivalent to vapor diffusion: namely, dialysis and ultrafiltration. In clinical manufacturing, large scale crystallization can be used as a purification or concentration step. Furthermore, in such operation ammonium sulfate could be replaced by other common sulfate salts.

The final concentration of the interferon alpha-2 in the sulfate salt solution at the point of crystallization, i.e., at the point of first crystal formation, can range from about 10 to about 80 mg/mL. More preferably, the concentration of interferon alpha-2 is from about 30 to about 50 mg/mL. Preferably, the interferon alpha-2 starting concentration is about 20 mg/ml.

The concentration of the sulfate salt in the interferon alpha-2 solution at the initial stage prior to the start of crystallization procedures can range from about 2% to about 18% saturation, i.e., the sulfate salt present at 2 to 18% of the concentration which would be present if the solution were 100% saturated. More preferably, the concentration of the sulfate salt is from about 7% to about 15% saturated in the interferon alpha-2 solution. In the counter solution at the start of the crystallization procedure, the concentration of sulfate salt is from about 12% to about 30% saturation, more preferably, from about 15% to about 25% saturation.

The pH of the interferon alpha-2 solution and the counter sulfate salt solution is preferably controlled in the range of from about 6.5 to about 8.5, more preferably from about 7.3 to about 8.0. Any suitable buffer can be employed for this purpose. For example, sodium phosphate, Tris[hydroxymethyl]-aminomethane hydrochloride or N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] buffers can be employed.

The crystallization preferably is performed under controlled temperature conditions. The temperature is preferably in the range of from about 15° to about 37°C, more preferably from about 18° to about 25°C.

Since the present invention surprisingly produces crystalline interferon alpha-2 at room temperature, it offers a distinct



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advantage over crystals obtained from polyethylene glycol at 4°C. Storage and formulation of human interferon-alpha 2 crystals at room temperature is now possible. Dissolution of the crystals can be accomplished by dialysis against 20mM sodium phosphate, pH 7.5  
5 buffer with 0.15 M sodium chloride added. Moreover, since sulfate salt solution is easier to remove, e.g., by dialysis, than polyethylene glycol, purer interferon alpha-2 crystals can be obtained upon redissolution with the method of the invention.

The crystalline interferon alpha-2 prepared by the method  
10 of the invention will form the basis for various pharmaceutical formulations. For example, the crystalline interferon can be employed in a slow release formulation, e.g. a depot preparation capable of releasing the equivalent of a daily dose of 0.1 - 1.0 µg/kg body weight. A depot preparation employing crystals prepared by the method of the  
15 inventions should exhibit considerably slower rate of dissolution than a formulation containing crystals produced at 4°C. In particular, ambient temperature (22°C) crystals should be less temperature sensitive than crystals that require a lower temperature of formation.

In addition, complexes of metals and human interferon  
20 alpha-2 can be formed then subsequently crystallized by the method of the invention. The crystals of such a complex could likewise be used in a slow release formulation. Examples of metal ion - protein complexes used in slow release formulation have been described in prior art. A complex of zinc and insulin prepared by the addition of zinc chloride to a  
25 sterile insulin solution was described in U.S. Patent 2,882,203 assigned to Novo Terapeutisk Laboratorium A/S. The crystalline suspension of such complex was 4 to 6 times longer acting than a non-crystalline preparation (Remington Pharmaceutical Sciences 17, Gennaro, A.R., ed., Mack Publishing Co., Easton, Penn. (1985) 975-976). The zinc-  
30 insulin complex is commercially available in different forms from several manufactures for example: Lente Insulin (Squibb), Ultra-Lente Iletin (Lilly) and Ultralente Insulin/Ultratard (Squibb-Novoo), Remington Pharmaceutical Sciences 17. Gennaro, A.R. ed., Mack Publishing Co., Easton, Penn. (1985) 975-976. Isophane Insulin is a crystalline product  
35 of insulin (zinc-insulin) and an alkaline protein (salmiridine). The

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product is manufactured under several trade names; NPH-Iletin (Lilly), Insulated (Leo) and Novolin N (Squibb-Novo) (Pharmaceutical Manufacturing Encyclopedia 1, Sittig. M. ed., (1988) 820-822). A zinc-interferon alpha-2 complex can be formed by methods described in U.S. Patent 2,882,203. The Interferon-alpha-2-zinc complex can be crystallized using the methods of this invention. The resulting crystalline suspension (comprising crystals of uniform size) formulated with appropriate additives (e.g. methylparaben, sodium chloride and sodium acetate) can be used for subcutaneous injection.

The crystalline interferon alpha-2 prepared by the method of the invention can be used in basically the same manner in which prior interferon alpha-2 materials have been used in pharmaceutical preparations, e.g., depot preparations of interferon alpha-2, which can be designed to administer a daily dose of about 0.1  $\mu\text{g/kg}$  to about 1.0  $\mu\text{g/kg}$  of crystalline interferon alpha-2. Such preparations contain a physiologically effective amount of the crystalline interferon alpha-2 in association with a conventional pharmaceutically acceptable carrier.

The invention disclosed herein is exemplified by the following working example, which should not be construed to limit its scope. Alternative methods within the scope of the invention will also be apparent to those skilled in the art.

The interferon alpha-2 employed was recombinant human interferon-alpha 2b expressed in *E. coli* as described in Weissmann, et al., *Science*, 209, 1343 (1980). The cells were cultured, harvested and extracted as previously reported in Leibowitz, P. et al (1982) US Patent 4,315,852. The resulting extracts was purified by a combination of conventional purification steps: ethanol extraction, matrix gel blue ligand affinity chromatography, ion exchange and gel filtration chromatography. The resulting purified interferon alpha-2b preparation was further purified by reversed-phase HPLC using a Rainin Auto Prep Chromatography system. Purified interferon alpha-2b (50mg) was chromatographed on a Rainin (4.1 x 250cm) C<sub>4</sub> 300 Angstrom column which had been pre-equilibrated with 27% acetonitrile, 0.1% trifluoroacetic acid (TFA). A linear gradient of 27%-72% acetonitrile, 0.1% TFA over a 30 minute period at 40ml/min was used to elute the

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interferon alpha-2b from the column. Eluted fractions were collected based on the absorbance profile from an in-line Knauer UV detector set at 280 nanometers.

Crystallization was achieved by vapor diffusion using  
5 either the hanging, sitting or sandwiched drop technique. Hanging drop vapor diffusion experiments were performed in 24 well tissue culture plates (Becton Dickinson and Company, Lincoln park, NJ). Sandwiched drop experiments were performed in protein crystallization plates CrystalPlate™ (Flow Laboratories, McLean, VA) and sitting drop vapor  
10 diffusion experiments were performed in MVD/24 multi-chamber vapor diffusion plates (Cryschem, Inc., Riverside, CA).

For hanging droplets, 10 µl droplets containing 20 mg/mL of protein in 10% aqueous saturated ammonium sulfate, 40 mM sodium phosphate, pH 8.0 were hung from siliconized coverslips inverted on  
15 Linbro tissue culture plates. These droplets were equilibrated against 1 mL of 20% saturated ammonium sulfate, 40 mM sodium phosphate, pH 8.0. Large monoclinic prismatic crystals (0.5 x 0.5 x 0.5 mm) were evident anywhere from 4-15 days of incubation at 22°C. Comparable  
20 solutions and experimental conditions were used for the sitting and sandwiched droplet experiments. The crystals were stable to X-ray diffraction analysis and diffracted to 6 Å resolution. Different batches of crystals were subject to X-ray analysis and gave consistent results with respect to morphology. This is the first report of an X-ray diffraction  
25 pattern for interferon alpha-2.

For X-ray studies, crystals are mounted in glass capillaries and are photographed with a precession camera at 22°C using CuKα radiation from a Rigaku RU-300 rotating anode generator operating at 40 kV and 100 mA. The native data set is collected on a Nicolet X-100A area detector using the same radiation source.

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## CHARACTERIZATION

### 1. BIOASSAY

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Individual crystals were extracted from a hanging droplets with a syringe, then resuspended in 100  $\mu$ l of wash solution consisting of 35% saturated ammonium sulfate, 40 mM sodium phosphate, pH 8.0 at 22° C. The suspension was centrifuged and the wash solution was removed with a Pasteur pipette. The washed crystals were redissolved in 100  $\mu$ l of 20 mM sodium phosphate, pH 7.5, 0.15 M sodium chloride, at 22°C.

Protein was determined by a modified Bradford assay using pure human Interferon alpha-2b as a reference standard. Antiviral activity was determined by a cytopathic inhibition assay using human foreskin diploid fibroblasts and encephalomyocarditis virus (ATCC-VR-129). A detailed description of the assay is provided in S. Rubinstein, P.C. Familletti and S. Pestka, J. Virol. 37 (1981) 755-758. The redissolved solution yielded a specific activity of  $2.0 \times 10^8$  lu/mg. This value is the same as that predicted for the original interferon alpha-2b preparation prior to crystallization, within the limits of the assay ( typically within the range  $1 \times 10^8$  to  $3 \times 10^8$  lu/mg).

## 2. HPLC

Analytical high performance liquid chromatography (HPLC) (Waters Ass. Milford, MA) was performed on an aliquot of redissolved interferon crystals. The sample was applied to a Rainin Dynamax C<sub>4</sub> 300 Angstrom column (4.6 x 250mm) which was subsequently eluted with a linear gradient of acetonitrile 27-72% in 0.1% trifluoroacetic acid over a 30 minute period. A Gilson variable wavelength detector set at 280nm with a sensitivity of 0.02 absorbance units was used to monitor the eluate. The retention times and chromatographic profiles of both the redissolved crystal solution and the original interferon alpha-2b preparation prior to crystallization were identical.

## 3. SDS-PAGE ANALYSIS

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A comparison was made of interferon alpha-2b before and after crystallization by the one-dimensional 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method described in Laemmli, U. K. (1970) Nature 227, 680. There was no variation in  
5 relative mobility when the samples were compared in parallel lanes on the same gel.

From 1, 2 and 3 above, there is clearly no reason to suppose that any chemical changes or any denaturing of the protein took place during the crystallization or reconstitution.

10 While the present invention has been described in conjunction with the specific embodiments set forth above, many alternatives, modifications and variations thereof will be apparent to those of ordinary skill in the art. All such alternatives, modifications and variations are intended to fall within the spirit and scope of the present  
15 invention.

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**WHAT IS CLAIMED IS:**

1. A method for the preparation of crystalline interferon alpha-2 which comprises equilibrating a sulfate salt solution containing  
5 interferon alpha-2 against a sulfate salt solution that will cause the interferon alpha-2 solution to become more concentrated and form Interferon alpha-2 crystals.
2. The method of claim 1, characterized by the  
10 equilibration being effected by means of ultrafiltration or dialysis, or by vapor diffusion.
3. The method of claim 1 or 2, characterized by the  
15 equilibration being effected by vapor diffusion using hanging or sandwich droplets.
4. The method of any of claims 1-3, characterized by a  
20 sulfate salt solution of interferon alpha-2 being equilibrated against a more concentrated sulfate salt solution.
5. The method of any of claims 1-4, characterized by the  
interferon alpha-2 is human, recombinant interferon alpha-2b.
6. The method of claim 5, characterized by the sulfate salt  
25 being selected from an ammonium, calcium, cadmium, potassium, lithium, magnesium or sodium salt.
7. The method of claim 5, characterized by the sulfate salt  
30 being ammonium sulfate.
8. The method of claim 5, characterized by the sulfate salt  
solution of interferon alpha-2 including a buffer.
9. The method of claim 8, characterized by the interferon  
35 alpha-2 solution being buffered to a pH of 7.3 to 8.0.

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10. The method of claim 9, characterized by the buffer being sodium phosphate.

5           11. The method of claim 10, characterized by the sulfate salt being present in the interferon alpha-2 solution in a concentration of from about 12% to about 30% saturated at the point of interferon alpha-2 crystal formation.

10           12. The method of claim 10, characterized by the sulfate salt being present in the interferon alpha-2 solution in a concentration of from about 15% to about 25% saturated at the point of interferon alpha-2 crystal formation.

15           13. The method for crystallizing interferon alpha-2 characterized by using crystals obtained by a process of claim 1 as seed crystals.

20           14. A depot formulation comprising crystals of interferon alpha-2 in combination with a pharmaceutically acceptable depot carrier.

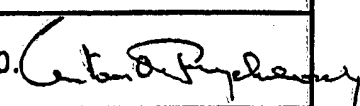
25           15. The formulation according to claim 14, characterized by the crystals of interferon alpha-2 being present in the form of a metal or protamine complex.

            16. The formulation according to claim 15, characterized by the metal complex being a zinc complex.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US. 91/03660

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C07K15/26 ; A61K37/66 ; C07K3/12 ; A61K9/22		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C07K ; A61K ; C12P	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	INTERFERON Y BIOTECNOLOGIA vol. 5, no. 3, September 1988, HAVANA-CUBA pages 286 - 287; A. DIAZ: 'LA CRISTALIZACION DEL INTERFERON ALPHA-2 RECOMBINANTE HUMANO ' see the whole document	14-16
A	---	1-13
Y	EP,A,281 299 (SCHERING CORPORATION) September 7, 1988 see claims 1-9	14-16
A	---	1-13
	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 262, no. 10, April 5, 1987, BALTIMORE US pages 4804 - 4805; S. VIJAY-KUMAR ET AL.: 'CRYSTALLIZATION AND PRELIMINARY X-RAY INVESTIGATION OF A RECOMBINANT FORM OF HUMAN GAMMA--INTERFERON ' see the whole document	
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
02 SEPTEMBER 1991	11.09.91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	RYCKEBOSCH A.O. 	



# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9103660  
SA 48198

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		AU-A- 1394688	26-09-88
		EP-A- 0375678	04-07-90
		JP-T- 3500286	24-01-91
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